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The goal of this cooperative agreement has been to generate, characterize and produce large quantities of purified monoclonal antibodies (mAb) that are endotoxin and aggregate-free. We have now perfected our large scale tissue culture techniques and scale-up HPLC Protein A affinity chromatography techniques suitable for the production of ≥200 mg quantities of mAb. Over 600 mg of purified mAb has already been supplied to the Army for research purposes, thus fulfilling the first specific aim of the project. We have also now generated and completely characterized a novel family of hamster mAb specific for either the p55 or p75 murine TNF receptors. Among these reagents are mAb which can either inhibit TNF activity (antagonist), or can alone, mimic TNF activity (agonist). This panel of reagents has provided the opportunity to trigger receptor-specific activities while avoiding unwanted or toxic reactions elicited with cytokine alone. We continue to investigate whether these reagents represent a therapeutic modality to permit desirable actions of a cytokine while at the same time preventing pathobiologic actions. Thus, we believe that we have achieved the initial goals of the project and continue to investigate the potential of these novel mAb.

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Robert D. Schmidt 1/24/96

PI - Signature Date

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I. INTRODUCTION

The general hypothesis on which this cooperative agreement was based is that unique monoclonal antibody reagents can be produced which can enhance or facilitate the actions of a particular cytokine so as to confer upon the host an increased nonspecific resistance to a variety of disease causing infectious agents such as viruses, bacteria and parasites. Alternatively, monoclonal antibody reagents could be produced which could selectively block specific pathobiologic responses to a given cytokine, while at the same time, allowing select beneficial reactions. To test this hypothesis, three specific aims were proposed. First, we planned to develop the capability of producing substantial amounts of several of our existing hamster anti-murine cytokine mAb under tightly controlled tissue culture conditions and then purify them to homogeneity in aggregate-free and endotoxin free-form. Moreover, we agreed to provide up to 400 mg of purified mAb each year to Army scientists for research purposes as needed. *These goals have been achieved.* In addition, we have now generated a series of monoclonal mouse anti-hamster Ig reagents to serve as secondary reagents necessary to facilitate detection and quantitation of our unique hamster mAb. These are the first monoclonal reagents of this type and should provide a much needed tool for future studies.

The second specific aim was to generate cytokine specific mAb reagents that enhance select cytokine activities in an attempt to augment the activity of endogenously produced cytokines elaborated during immune or inflammatory response and thereby promote host defense. *To address this specific aim we generated and characterized a family of mAb specific for the murine TNF receptors. This panel of receptor specific reagents include both antagonistic and agonistic mAb. One particular mAb can drive TNF reactions mediated through the p55 receptor both in vitro and in vivo in the absence of any p75 initiated responses.*

Finally, the third specific aim was to produce monoclonal antibody reagents that selectively inhibit only certain biologic activities of a given cytokine but not other activities and thereby promote host defense while at the same time ablate toxic or immunopathologic effects. *Towards these goals the above mentioned panel of TNF receptor specific mAb has been particularly informative in dissecting the positive versus negative effects of this given cytokine.* Thus, throughout the course of this cooperative agreement we have significantly advanced our original hypothesis and have developed several novel reagents for use in both in vitro and in vivo studies. Our findings to date demonstrate that, at least in the analysis of TNF-mediated events, the use of receptor specific reagents may indeed provide a mechanism to selectively promote host defense.

II. NARRATIVE OF PROGRESS

A. Specific Aim 1

We have fulfilled the first objective of this aim and now have the capability to generate within the lab substantial quantities of highly purified, contaminant free mAb. We had previously generated and characterized a family of hamster hybridomas that produced neutralizing mAb against murine IFNγ, TNFα and IL-1 (1-5). These hamster mAb have the advantage of interacting with antigen in a specific, ahigh affinity manner, display a relatively long circulatory half life and are non-immunogenic in mice, making them excellent candidates for in vivo studies aimed at dissecting the roles of these agents in promoting host defense. We began our studies by developing large scale production protocols for both the IFNγ and TNF reactive hybridomas.

Two procedures were used to generate large quantities of antibody in crude form that were suitable for subsequent purification. The first was based on the conventional protocol of generating antibody containing culture supernatants in roller bottles. We adapted our hybridoma cell lines to grow in roller bottles and then recloned them to isolate high antibody producers. Then each cell line was expanded into 20 liters of medium and grown to stationary phase. Depending on the particular cell line, the spent culture supernatants were found to contain between 10-30 mg mAb per liter, thus permitting us the capacity to generate between 100-500 mg of each antibody in crude form in approximately 4 weeks. The second protocol was based on the use of Amicon Mini Flo-Path Bioreactors. Several hybridoma lines were adapted to the specialized WRC medium used for growth in the Bioreactors. All cultures of hamster hybridomas required supplementation of WRC medium with 5% FCS. Two of the units tested were extremely successful and have yielded 600 and 700 mg of crude antibody, respectively. The third unit developed a leak early in the run and therefore only 150 mg of antibody was obtained. Compared to conventional roller bottle cultures, the Bioreactors had the advantage of producing supernatants with higher (30-100 times greater) antibody concentrations and smaller volumes generated prior to purification (10 ml per day at 0.3-1.0 mg/ml). However, the generation of large quantities of antibody required the unit to run for extended periods of time (longer than needed to generate similar quantities in roller bottles). The use of Bioreactors does not provide a significant cost savings in the large scale production of antibodies due to the cost of the Bioreactor unit itself and the specialized medium used in these units. Moreover, the model tested in these studies has now been discontinued necessitating an evaluation of new models if this system is to be used in the future. Thus, we have continued to produce large scale preparations of crude antibody containing supernatants using roller bottles.

Large scale antibody purification has been achieved using a 5.0 x 12.0 cm column packed with Protein A Affiprep (Bio-Rad Laboratories, Richmond, CA) under endotoxin free conditions. If necessary antibody containing culture supernatants are concentrated in an Amicon spiral concentrator, cleared by centrifugation and then pumped onto the Protein A Affiprep column equilibrated in 0.05M borate buffer/0.15M NaCl using a Waters 650 HPLC system adapted with a peristaltic pump for sample application. The column is washed with 10 volumes borate buffer and then antibody is eluted using 0.2 M glycine-HCl/0.15M NaCl pH 2.0. (In cases where the particular antibody is prone to form aggregates, the column is eluted with a linear gradient of glycine over one column volume.) The fractions are collected into plastic tubes containing 1.0M Tris buffer pH 8.5 and analyzed for protein by monitoring absorbence at 280 nm. Antibody-containing fractions are pooled, dialyzed, sterile filtered and frozen. We now routinely use this protocol to produce 200-400 mg quantities of purified antibody per run.

The development of this antibody production protocol allowed us to meet our commitment to supplying Army researchers with highly purified cytokine specific monoclonal antibodies. In addition, we have now produced a control hamster mAb (L2-3D9) which does not react with any naturally produced murine cytokine (6). The specific antibodies delivered are as follows:

ANTIBODY	SPECIFICITY	QUANTITY	DATE SHIPPED
H22	Murine IFNγ	100 mg	9/92
TN3-19.12	Murine TNFα	100 mg	9/92
H22	Murine IFNγ	125 mg	7/93
TN3-19.12	Murine TNFα	125 mg	7/93
L2-3D9	Control Ig	42 mg	2/95
H22	Murine IFNγ	120 mg	9/95
Total Antibody		**612 mg**	

There has been very little characterization of Armenian hamster immunoglobulin subtypes in the literature. Our own survey of the purified hamster mAb generated in our laboratory has revealed at least three subclasses of protein A binding Ig based on heavy chain size. During the course of the development, production and analysis of our unique panels of hamster monoclonal antibodies reactive with murine proteins (cytokine and cytokine receptors), it became obvious that the secondary reagents

commercially available to detect, immunoprecipitate or quantitate these mAb were of variable quality, specificity and affinity. In addition, nearly all of the polyvalent reagents available were highly contaminated with endotoxin. To circumvent these difficulties we decided to develop our own monoclonal anti-hamster Ig reagents. To date two series of murine monoclonal antibodies reactive with hamster mAb have been generated. The first series of mouse anti-hamster Ig reagents produced (MAH) reacted with only the most common heavy chain form of hamster Ig. MAH 1.12 (mouse IgG_{2b})is capable of detecting the "appropriate" hamster mAb in ELISA, FACS and Western Blot analysis. Moreover, MAH1.12 did not crossreact with either murine, rat or rabbit Ig. However, MAH1.12 is unable to recognize several of the commonly used hamster mAb (B122) (3) and would also be inappropriate to use as a secondary reagent for screening additional fusions as many potential positive hybridomas would not be identified. We therefore immunized additional animals with a combination of hamster mAb representing members of all the hamster Ig subclasses. The newly generated mouse anti-hamster Ig hybridomas (HAMS) appear to react with epitopes common to all of the hamster mAb currently in use when assayed by ELISA. We continue to characterize the HAMS mAb and are currently expanding these clones for large scale production. These purified, endotoxin-free, high affinity mAb (HAMS 8G1 and 1H6) should be extremely useful in both screening assays and in in vitro biologic assays where antibody crosslinking is desired.

B. Specific Aim 2

Our second goal was to explore whether we could produce a monoclonal antibody capable of **enhancing** the actions of a cytokine. The initial plan was to focus on an antibody (H1.5) that we had already produced that appeared to enhance the action of IFNγ in vitro (1). However, at this same time we identified a novel mAb reactive with a cytokine receptor (the murine p55 TNF receptor) that had agonist activity and could thereby mimic a specific cytokine action. TNF exhibits a broad range of biologic activities (protective and pathobiologic) signaled through the binding of trimeric ligand to either of two cell surface receptors of Mr 55 Kd and 75 Kd, respectively. TNF is a critical mediator in promoting host defense to bacterial agents, yet, can also have lethal effects, as in the case of endotoxin shock (2,7-9). We chose to examine the modulation of TNF mediated responses and have now generated both agonist and antagonist mAb specific for each of the two TNF receptors (10).

Appendix 2 contains the published characterization of these TNF receptor specific mAb (10). These are extremely unique reagents since at the present time they represent the only mAb in existence that can detect the murine receptor proteins. The p55 agonist mAb 55R-593 was capable of

inducing specific biologic activity both in vitro and in vivo. The p55 TNF receptor specific mAb 55R-593 alone could induce cell lysis, provide a second signal for nitric oxide induction (Fig. 1), trigger NF-kB activation (Fig. 2) and in vivo can stimulate the shedding of the p75 TNF receptor in a fashion similar to human TNF (Fig. 3). Moreover, the TNF receptor specific mAb circulate in vivo with a half life of nearly three days, which far exceeds the clearance of recombinant murine or human TNF, which have a half life measured in minutes. Thus, it appears that 55R-593 may be useful in initiating events signaled through the p55 receptor in an efficient manner. In addition, in vivo treatment with murine TNF has the problem of producing serious tissue necrosis at the sight of injection. This reaction requires the engagement of both the p55 and p75 TNF receptors. Therefore, stimulation of the p55 receptor alone (using 55R-593) can successfully induce particular reactions without causing local tissue destruction.

C. Specific Aim 3

In the third specific aim we determined whether we could produce a therapeutic modality that will permit the desirable actions of a cytokine while at the same time inhibit its pathobiologic actions. We chose to use our family of novel TNF receptor specific mAb in this effort. Several neutralizing mAb reactive with either the p55 or p75 TNF receptor were identified (Appendix 2). Blockade of either the p75 or p55 TNF receptors were effective in preventing ligand induced skin necrosis. Moreover, the p55 reactive, antagonist mAb (55R-170 and 55R-176) could inhibit TNF dependent cell lysis, block the induction of nitric oxide synthase and protect mice from the lethal effects of endotoxin shock. However, neutralization of TNF activity had profound effects during the course of bacterial infection. Treatment of mice with antagonistic p55 reactive mAb became susceptible to sublethal challenge with *Listeria monocytogenes* and died. Thus, it is important to consider all aspects of the function of a particular cytokine to optimize the benefit of blocking the action(s) of that given mediator. The p75 reactive mAb were without functional consequence in models of endotoxin shock or bacterial challenge.

As part of the characterization of this family of receptor specific mAb, we were able to develop an ELISA capable of quantitating picogram quantities of soluble receptor present in the serum of both normal animals and those challenged with LPS. Epitope mapping studies identified non-competing pairs of p55 and p75 TNF receptor specific hamster mAbs that were subsequently used to develop TNF receptor specific ELISAs. These ELISAs were not altered by the presence of up to 1 μg/ml of exogenously added MuTNF (data not shown). Ninety minutes after LPS challenge, TNF levels reached 9 ng/ml and then rapidly declined as previously described (Fig. 4A). In these same samples, LPS induced shedding of the p75 TNF receptor which reached maximum serum levels of 20 ng/ml 8 hours after challenge and

remained elevated for several hours (Fig. 4C). Appearance of soluble p55 was more rapid (peak levels were obtained 30 minutes after LPS challenge) but was of lower magnitude (peak value of 1.4 ng/ml) (Fig. 4B). These results thus establish that the p55 and p75 specific ELISAs are useful in quantitating soluble forms of TNF receptors present in serum.

In vivo administration of antibodies specific for the TNF receptors resulted in the substantial linear accumulation of both types of soluble receptors in serum by binding and preventing their clearance (Fig. 5). Having determined that both TNF receptors are continually shed, we wanted to ascertain the participation of each receptor in the binding and clearance of TNF in response to inflammatory stimulus. Non-blocking antibodies to the p55 or p75 were used to immunoprecipitate soluble receptors in the serum of mice treated with or without LPS. The soluble forms of p55 and p75 of Mr 28 and 40, respectively, were both immunoprecipitated from the serum of mice treated with LPS (data not shown). Both p55 and p75 TNF receptor specific mAbs co-immunoprecipitated TNF, demonstrating that both receptors are capable of binding to TNF in vivo and inhibiting its bioactivity. It may be that the high rate of constitutive p55 shedding (which has been previously unobserved) serves as a non-specific, stimulus-independent mechanism for clearing TNF in vivo. Thus, stabilization of naturally produced soluble TNF receptors may also serve as a therapeutic modality for neutralization of TNF activity.

III. Conclusions

As we approach the end of this cooperative agreement we can safely state that we have been successful at completing each of our goals. We have generated and characterized an extensive panel of cytokine and cytokine receptor specific monoclonal antibodies. In addition, we have produced important control antibodies and secondary reagents necessary for the detection and quantitation of our unique hamster mAb. The development of large scale antibody purification protocols has allowed us to produce 200-400 mg quantities of mAb in a single run which is both free of aggregates and endotoxin, and is therefore suitable for sensitive in vivo experiments. Greater than 600 mg of these mAb have been provided to Army researchers. Using our newly developed hamster mAb specific for the murine TNF receptors, we have begun to dissect the role of each receptor in mediating TNF's pleiotropic actions. We have begun to capitalize on the receptor-specific signaling of certain TNF actions using our mAb that can act either to induce/agonize or prevent/antagonize individual responses. We have identified a p55 agonist mAb capable of inducing by itself several TNF mediated actions, without inducing certain pathobiologic responses (tissue necrosis). Antagonistic mAb reactive with both p55 and p75 have also been identified. The anti-p55 reagents are particularly useful in blocking TNF-mediated lethal shock. In addition, we have developed an ELISA to quantitate soluble receptors levels in the serum. Administration of non-blocking mAb can stabilize levels of soluble receptor in the serum, which may act as important buffers in controlling TNF mediated activities in vivo. We continue to examine the potential of these reagents to either enhance or inhibit cytokine actions in order to promote host defense and at the same time prevent pathobiologic reactions.

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APPENDIX 1

(Figures and Legends)

Figure 1: *Induction /Inhibition of Nitric Oxide Production by Anti-p55 and Anti-p75 mAb.* Dilutions of anti-p55, anti-p75 or the 6C8 control hamster mAb were incubated with L929 cells (6×10^4/well) for 2 hours at 37°C prior to addition of MuIFNγ alone (Panels A and B) or in the presence of either 300 pg MuTNFα (Panels C and D) or 2000 pg HuTNFα (Panels E and F). Supernatants were harvested after 48 hours and nitrite levels quantitated using Greiss reagent. Stimulation of L929 cells with MuTNF or HuTNF in the absence of mAb produced 2.37 and 2.76 nmoles nitrite, respectively.

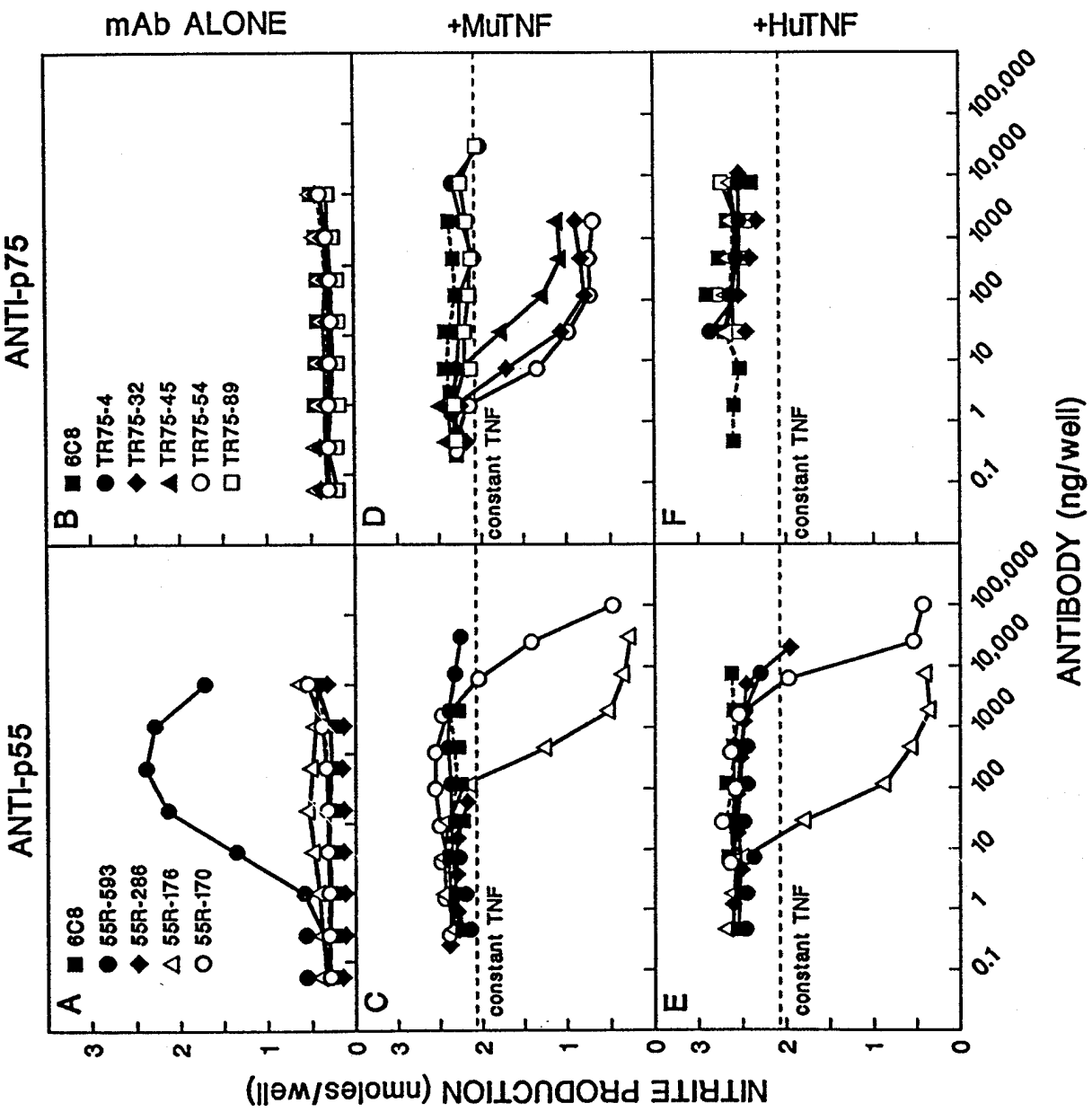
Figure 2: *NF-kB Activation by p55 Engagement.* Meth A tumor cells (1×10^6) were incubated with different concentrations of either human TNFα or 55R-593 for 2 hours. Nuclear extracts were prepared and electrophoretic mobility shift assays were performed using a ^{32}P-labeled probe from the Igκ enhancer. Bands representing activated NF-kB were quantitated by densitometry.

Figure 3: *55R-593 Induction of p75 Shedding In Vivo.* Mice were injected with 300 μg HuTNF or 500 μg mAbs specific for p55 and bled at 3 hours. Serum was then assayed for p75 by ELISA. The constitutive amount of p75 was subtracted such that the graph represents TNF induced shedding of p75. Data presented are the means of 3 mice per group.

Figure 4 *TNF, p55 and p75 levels in Balb/cByJ mice after i.p injection of 0.6 mg LPS.* Groups of 5 mice were injected with 0.6 mg LPS and bled at various timepoint. Serum was pooled and assayed for ELISA for **A:** TNF; **B:** p55; or **C:** p75.

Figure 5: *Accumulation of Soluble TNF Receptors after Administration of TNF Receptor mAbs in vivo.* Groups of 3 mice were treated with 250 μg of control mAb or non blocking anti-TNF receptor mAbs (55R-286 or TR75-89). At various time intervals mice were bled and the serum was assayed for the presence of soluble p55 and p75 by ELISA. The linear regression of the data are shown.

Figure 1


mAb ALONE
+MuTNF
+HuTNF
ANTI-p75
ANTI-p55
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● 55R-593
◆ 55R-286
△ 55R-176
○ 55R-170
B ■ 6C8
● TR75-4
◆ TR75-32
▲ TR75-45
○ TR75-54
□ TR75-89
constant TNF
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ANTIBODY (ng/well)
0.1
1
10
100
1000
10,000
100,000

Figure 2

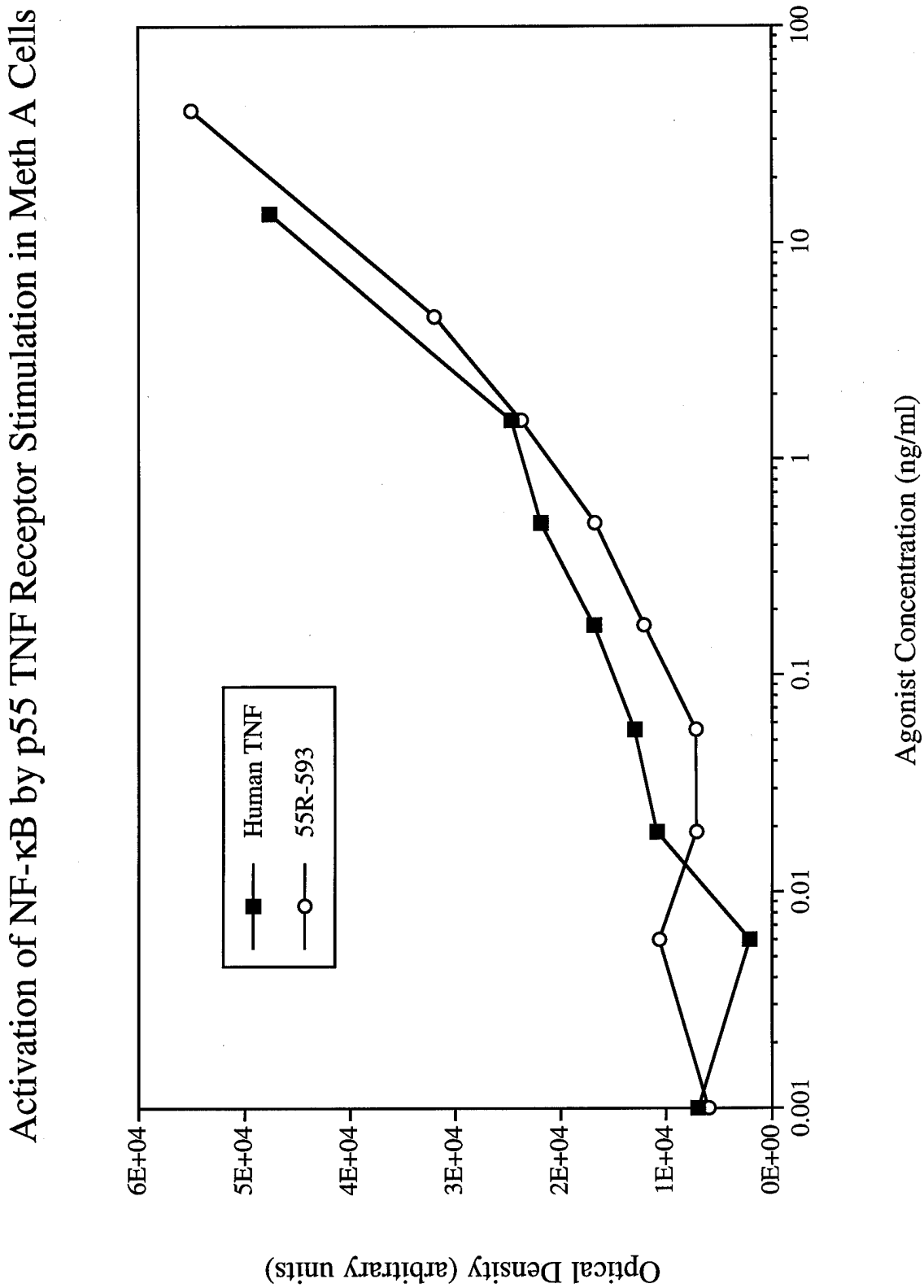
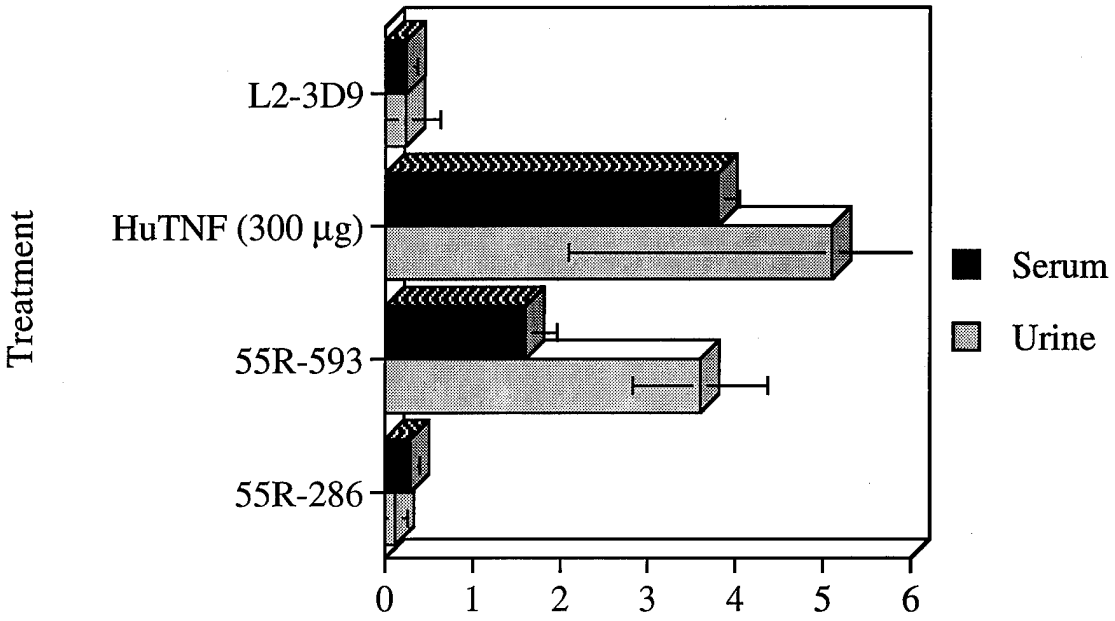

Activation of NF-κB by p55 TNF Receptor Stimulation in Meth A Cells
Optical Density (arbitrary units)
6E+04
5E+04
4E+04
3E+04
2E+04
1E+04
0E+00
0.001
0.01
0.1
1
10
100
Agonist Concentration (ng/ml)
Human TNF
55R-593

Figure 3

Induction of p75 Shedding in vivo



Kinetics of TNF, p55 and p75 levels in serum after ip injection with LPS

Figure 4

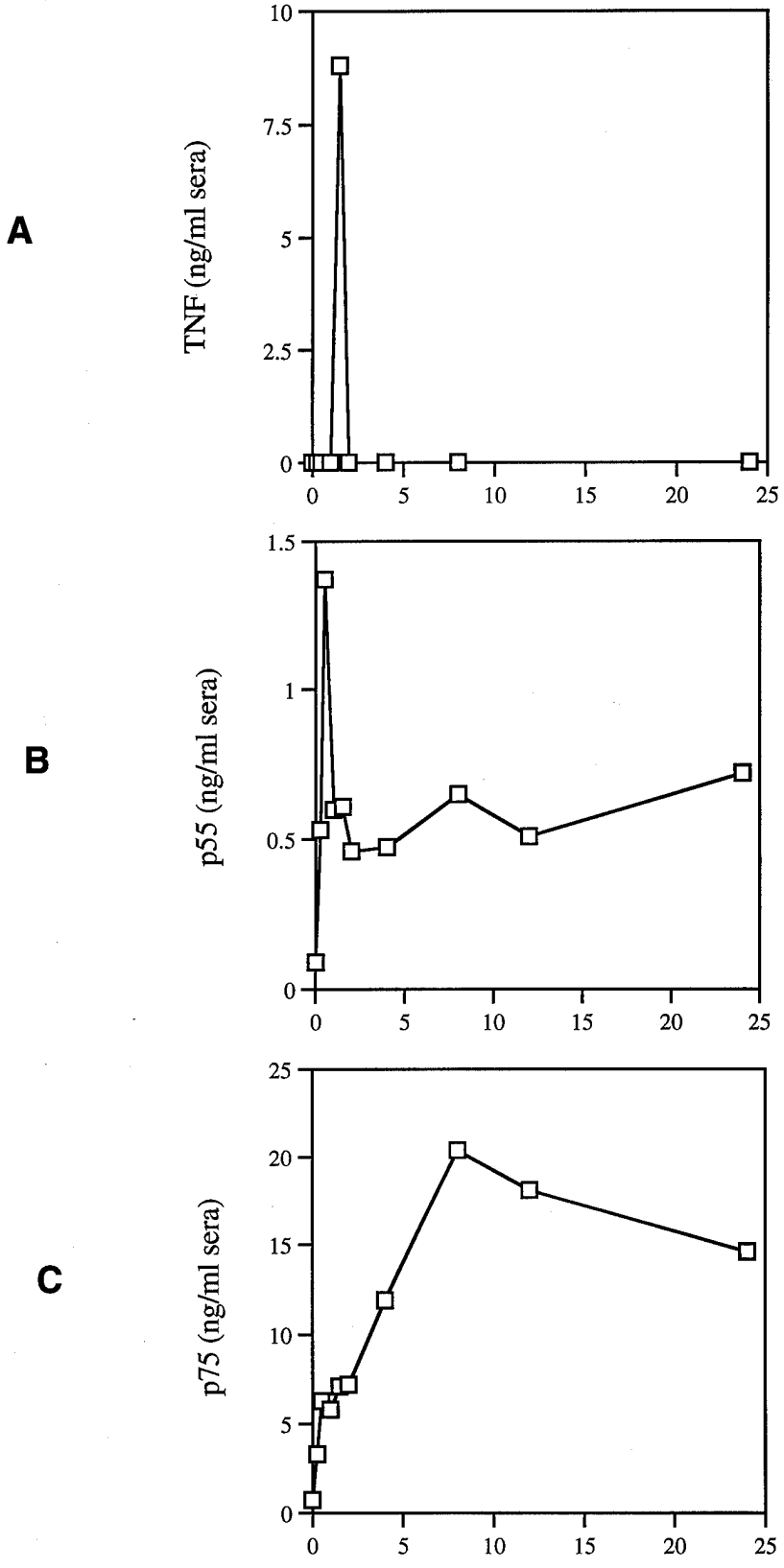
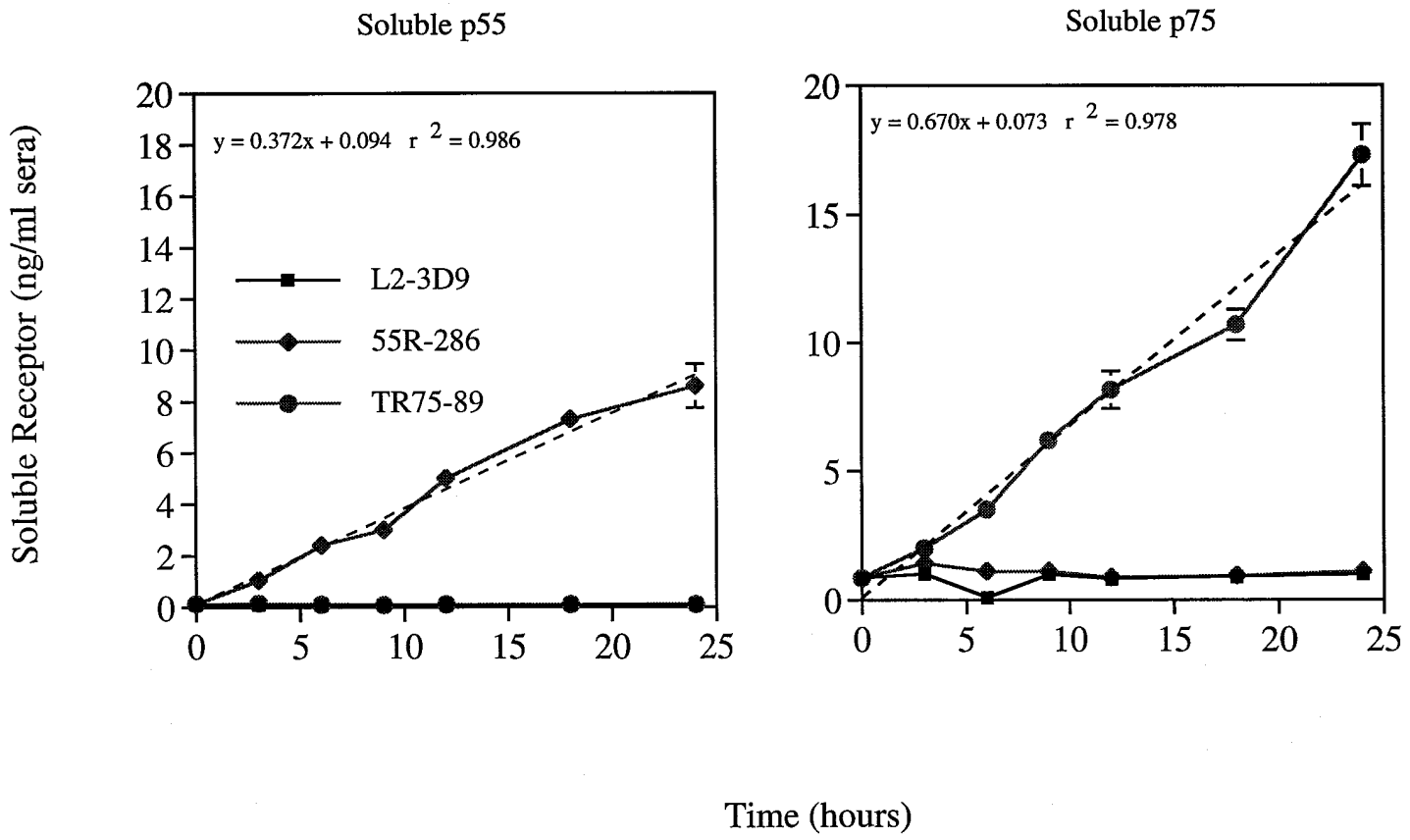


Figure 5

Appearance of Soluble TNF Receptors after administration of TNF Receptor mAbs in vivo



APPENDIX 2

(Reprint)

Monoclonal Antibodies Specific for Murine p55 and p75 Tumor Necrosis Factor Receptors: Identification of a Novel In Vivo Role for p75

By Kathleen C. F. Sheehan, J. Keith Pinckard, Cora D. Arthur, Louis P. Dehner, David V. Goeddel,* and Robert D. Schreiber

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Summary

Monoclonal antibodies (mAbs) specific for the murine p55 and p75 tumor necrosis factor (TNF) receptors were produced after immunization of Armenian hamsters with the purified soluble extracellular domains of each receptor protein. Four p55- (55R) and five p75 (TR75)-reactive mAbs immunoprecipitated the appropriate receptor from the surface of L929 cells. None of the mAbs cross-reacted with the other TNF receptor form. The mAbs were functionally characterized by their ability to inhibit ligand binding and influence TNF-dependent L cell cytolytic activity or proliferation of the murine cytolytic T cell clone CT6. One p55-specific mAb, 55R-593, displayed agonist activity, while two other p55-specific mAbs (55R-170 and -176) were found to be TNF antagonists. The fourth mAb (55R-286) had no functional effects on cells. Several antibodies specific for the p75 TNF receptor partially inhibited recombinant murine TNF-α–dependent cytolytic activity (60%). Blocking mAbs specific for p75 but not anti-p55 inhibited TNF-mediated proliferation of CT6 T cells. When used in vivo, p55- but not p75-specific mAbs protected mice from lethal endotoxin shock and blocked development of a protective response against *Listeria monocytogenes* infection. In contrast, both p55 and p75 mAbs individually blocked development of skin necrosis in mice treated with murine TNF-α. These data thus demonstrate the utility of the two families of murine TNF receptor–specific mAbs and identify a novel function of the p75 TNF receptor in vivo.

TNF, originally identified by its ability to effect hemorrhagic necrosis of specific tumors (1), is now known to play a major role in promoting immunologic, inflammatory, and pathobiologic reactions (2). TNF's pleiotropic activities are induced after binding of the homotrimeric ligand to either of two distinct cell surface receptors of 55 and 75 kD that are independently expressed on a variety of different cell types (3, 4). The cDNAs and genes for the murine and human p55 and p75 TNF receptors have been cloned and expressed and the proteins they encode characterized (5–9). Although the extracellular domains of the two TNF receptors are 28% identical, no homology has been found between the intracellular domains of the two proteins (10). This observation suggests that the two receptors may be responsible for inducing distinct cellular responses. The murine p55 TNF receptor binds both murine and human TNF-α. In contrast, the murine p75 receptor binds only its homologous murine ligand and not the human homologue (8, 9).

Work is ongoing to define the functional roles of the p55 and p75 TNF receptors on various cell types. The p55 receptor is now known to be important for inducing in cells a wide variety of functions such as cytolytic activity (11, 12), antiviral activity (13), expression of manganous superoxide dismutase (14) and intercellular adhesion molecule (ICAM)[1] 1 (15, 16), IL-6 mRNA accumulation (17, 18), and NF-κB induction (12, 15, 19, 20). Recent experiments using p55-deficient mice have demonstrated that the p55 TNF receptor plays a physiologically important role in promoting lethal shock induced by LPS and galactosamine and in effecting antimicrobial responses to *Listeria monocytogenes* (21, 22). In contrast, little information is available concerning the biologic responses mediated by the p75 TNF receptor. In vitro experiments have suggested that p75 enhances p55-induced biologic responses by facilitating binding of TNF to p55 (23, 24). In addition, p75 induces proliferative responses in certain cells of hematopoietic origin and enhances expression of certain adhesion molecules such as ICAM-1 (11, 14, 25–27).

[1] *Abbreviations used in this paper:* HuTNF, MuTNF, and MuIFN, recombinant human and murine TNF-α and IFN-γ, respectively; ICAM, intracellular adhesion molecule.

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Although receptor-specific mAbs have been generated against the human TNF receptors, no mAbs are currently available that are reactive with the murine TNF receptor proteins. Herein, we report the production and characterization of hamster mAbs specific for the murine p55 and p75 TNF receptors. The functional activities of these antibodies are demonstrated in well-defined in vitro systems and subsequently by use of in vivo murine models of lethal shock and anti-*Listeria* responses. Finally, using an in vivo model of TNF-induced skin necrosis in mice, we identify a novel physiologic role of p75.

Materials and Methods

Animals. C57Bl/6J, CBA/J, BALB/cByJ, and C3H/HeJ female mice 6–12 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in microisolator cages. Male Armenian hamsters were obtained from Cytogen Research and Development, Inc. (Cambridge, MA). Goats were purchased through and maintained at the Washington University School of Medicine Tyson Research Center.

Reagents and Materials. Media, supplements, and buffers used in these experiments were purchased or prepared as previously described (28, 29) and contained <0.005 endotoxin units/ml as determined by the *Limulus* amebocyte lysate assay (BioWhittaker Inc., Walkersville, MD). Purified recombinant murine TNF-α (MuTNF) (1.2×10^7 U/mg), recombinant human TNF-α (HuTNF) (5.6×10^7 U/mg) and recombinant murine IFN-γ (MuIFN) (4.7×10^6 U/mg) were produced by Genentech, Inc. (South San Francisco, CA). MuTNF was radiolabeled to a specific activity of 10.8 μCi/μg by use of $Na^{125}I$ (ICN Biomedicals, Inc., Irvine, CA) and Iodobeads (Pierce Chemical Co., Rockford, IL) according to the manufacturers' directions. Iodinated HuTNF (800 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Purified soluble extracellular domains of the p55 and p75 murine TNF receptors were prepared as described (8, 30) and radioiodinated by use of the Bolton-Hunter reagent (ICN Biomedicals, Inc.) according to the manufacturer's directions to a specific activity of 3.2 and 9.2 μCi/μg, respectively. LPS (*Escherichia coli* strain 0111:B4) and D-galactosamine were obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies. The following previously described and characterized hamster mAbs were used in this study: TN3-19.12 (anti-murine TNF-α) (29), JAMA-147 (anti-murine type I IL-1 receptor) (31), and H22 (anti-murine IFN-γ) (28); L2-3D9 (29) and 6C8 (32) are control hamster mAbs not reactive with naturally occurring murine proteins. Polyvalent rabbit p55- and p75-specific antisera were produced as previously described (11). Anti–hamster IgG was produced by immunization of goats with purified Armenian hamster IgG. Hamster IgG–specific antibodies were purified from immune sera purified by affinity chromatography by use of agarose-bound Armenian hamster IgG. Purified antibodies were biotinylated by use of the Enzotin reagent (Enzo Biochemical, Inc., New York) according to the manufacturer's instructions.

Production of Anti-TNF Receptor Antibodies. Hamster mAbs were generated from Armenian hamsters as previously described (28, 29). Animals were immunized intraperitoneally with the soluble purified extracellular domains of either the p55 or p75 murine TNF receptor (10 μg) emulsified in CFA and boosted three times with the same quantity of antigen in IFA at 2-wk intervals. 3 wk after the last boost, each animal was given an i.v. injection of 10 μg of the appropriate antigen diluted in sterile saline. Cultures were screened by immunoprecipitation of radiolabeled soluble receptor protein (2 ng/well) as previously described (33).

For the anti-p55 fusion, 565/612 growth-positive wells were obtained, which yielded 20 separate cultures producing p55-specific antibody (3.54%). The p75 fusion produced 236/408 growth-positive wells, and 12 produced p75-specific antibodies (5.08%). Antibody-producing hybridomas were cloned by limiting dilution. mAbs were purified from clone culture supernatants by use of Affi-Prep Protein A Silica (Bio-Rad Laboratories, Richmond, CA) chromatography as previously described (29) and HPLC gel filtration. All antibody stocks contained <200 pg endotoxin per milligram of protein when analyzed by the *Limulus* amebocyte lysate assay.

Ligand Binding Inhibition. The ability of anti-p55 and anti-p75 mAbs to block ligand binding to L929 was determined by use of a protocol similar to that described previously (34). 4 million L929 cells were preincubated for 1 h at 4°C with serial dilutions of mAb and then incubated with radiolabeled recombinant TNF in a final total volume of 200 μl. To monitor binding of TNF to p55, 4 ng ^{125}I-labeled human TNF was used in the experiment since human TNF binds only to murine p55 TNF receptors (8). To monitor binding of TNF to p75 TNF receptors, cells were first incubated with 5 μg unlabeled human TNF for 1 h at 4°C to block the p55 TNF receptor, exposed to mAb, and then incubated with 20 ng ^{125}I-labeled murine TNF. The level of nonspecific binding in each experiment was determined by adding a 100-fold excess of unlabeled human or murine TNF to control wells. Cell-associated and free radioactivity were separated by centrifugation over a phthalate oil mixture (34) and the percent inhibition of specific binding determined.

Immunoprecipitation, SDS-PAGE, and Immunoblotting. L929 cells (2×10^7) were lysed in 1 ml PBS containing 1% Triton X-100 and protease inhibitors as previously described (35). Samples were precleared by 1-h incubation with 50 μl protein A–Sepharose slurry (Pharmacia Fine Chemicals, Piscataway, NJ) and then incubated overnight at 4°C with 20 μg mAb. 50 μl of protein A–Sepharose was added to each sample and incubated an additional 4 h. Beads were pelleted by centrifugation and washed three times with 1 ml immunomix (35) and once with 1 ml PBS. Beads were resuspended in 70 μl of Laemmli sample buffer (36), boiled 10 min, and centrifuged.

Half of each sample was applied to a 12% polyacrylamide gel and subjected to SDS-PAGE according to the method of Laemmli by use of a mini-Protean II gel apparatus (Bio-Rad Laboratories) (36). Western blot analysis was performed as previously described (35). Filters were washed in PBS containing 0.05% Tween-20 and incubated 2 h with biotin-conjugated anti-p55 (55R-593) or anti-p75 (TR75-54) at 2 μg/ml. After washing, filters were incubated 15 min with peroxidase-conjugated streptavidin (1:4,000), washed, and developed by use of the enhanced chemiluminescence system (ECL; Amersham).

L929 Cytolytic Assay. TNF-dependent L929 killing activity was quantitated as previously described (29). Briefly, L929 cell cultures (80% confluent) were harvested in EDTA, washed, resuspended to 7.5×10^5 cells/ml in medium, and treated with actinomycin D (2 μg/ml final concentration). After 2 h at 37°C, 100 μl of the cell suspension was mixed with 100 μl of serial dilutions of either mAb or TNF in 96-well flat-bottom culture plates. In assays in which cell-bound mAb was cross-linked, 20 μg of affinity-purified goat anti–hamster Ig was added per well. After 18 h of culture at 37°C, viability of the cell cultures was determined by vital dye staining with crystal violet as previously described (29).

Neutralization of TNF Activity. Neutralization of TNF-dependent cytolytic activity by mAb was quantitated by use of a modi-

fication of the TNF bioassay described above. Different concentrations of mAb were preincubated with actinomycin D–treated L929 cells (7.5×10^4/well) for 2 h at 37°C. A constant amount of TNF was then added to each well and the cultures incubated an additional 18 h at 37°C. Cell viability was assessed by crystal violet staining.

Nitric Oxide Assay. Nitric oxide induction in murine L929 fibroblasts by MuIFN and TNF was assayed essentially as described previously (37, 38).

CT6 Proliferation. TNF-dependent proliferation of the murine CT6 cytotoxic T cell line was assayed as previously described (11). For inhibition studies, cells were preincubated with mAb for 2 h at 37°C before addition of 2 ng/well MuTNF. Cultures were pulsed with [^{3}H]thymidine (0.5 μCi/well) during the final 4–6 h of a 24-h culture period and cpm incorporated into DNA determined.

LPS-Galactosamine-induced Shock. Groups of 10 C57Bl/6J mice were injected intraperitoneally with either pyrogen-free saline or mAb (250 μg) 24 h before treatment with a combination of 600 ng LPS and 10 mg D-galactosamine (intraperitoneally in 0.5 ml). Survival was monitored at 4- to 6-h intervals over a period of 72 h (39).

Listeria Infection. Mice were pretreated with either saline or mAb injected intraperitoneally 6 h before i.p. administration of 3,000 CFU of *Listeria monocytogenes* as described (40). Survival was monitored twice daily over a 2-wk period.

TNF-mediated Skin Necrosis. Mice were pretreated with either pyrogen-free saline or mAb (100 μg i.p.), anesthetized with sodium pentobarbital (80 mg/kg), and the hair removed from the back. 24 h later, the animals were injected subcutaneously with either saline, MuTNF (3 μg), HuTNF (3 or 30 μg), or a mixture of 3 μg TNF and mAb (50 μg) in 0.2 ml. Injections were continued on a daily basis. Mice were observed twice daily for the appearance of local skin hemorrhage and necrosis. At various times after treatment, the mice were bled, killed, and tissue prepared for histology according to standard procedures.

Results

Immunoprecipitation of Receptor from Cell Surfaces. Purified mAbs were examined for their ability to specifically precipitate intact receptor from cell surfaces. Western blot analysis showed that three of the anti-p55 mAbs (55R-593, -176, and -170) precipitated a protein of the appropriate molecular mass from L929 cell lysates (Fig. 1, *top*). In a separate experiment, similar results were obtained with a fourth p55-specific mAb, 55R-286 (not shown). No p55 was precipitated by any of the anti-p75 mAbs (TR75-32, TR75-45, or TR75-54) or control hamster mAbs specific for the murine type I IL-1 receptor (JAMA-147) or TNF-α (TN3-19.12). Conversely, the p75 receptor was precipitated only by mAb generated against the murine p75 TNF receptor extracellular domain (Fig. 1, *bottom*). In this case, Western blot analysis revealed that the anti-p75 mAb precipitated two components, a major component of 70 kD and a minor component of 60 kD. Similar patterns were seen with two additional p75-specific mAbs, TR75-4 and TR-75-89 (data not shown). No TR75-reactive bands were identified after precipitation with either the anti-p55 mAb or control IgG. The antigenic specificity of each mAb was verified by an ELISA that used immobilized purified p55 or p75 extracellular domain (data not shown). These

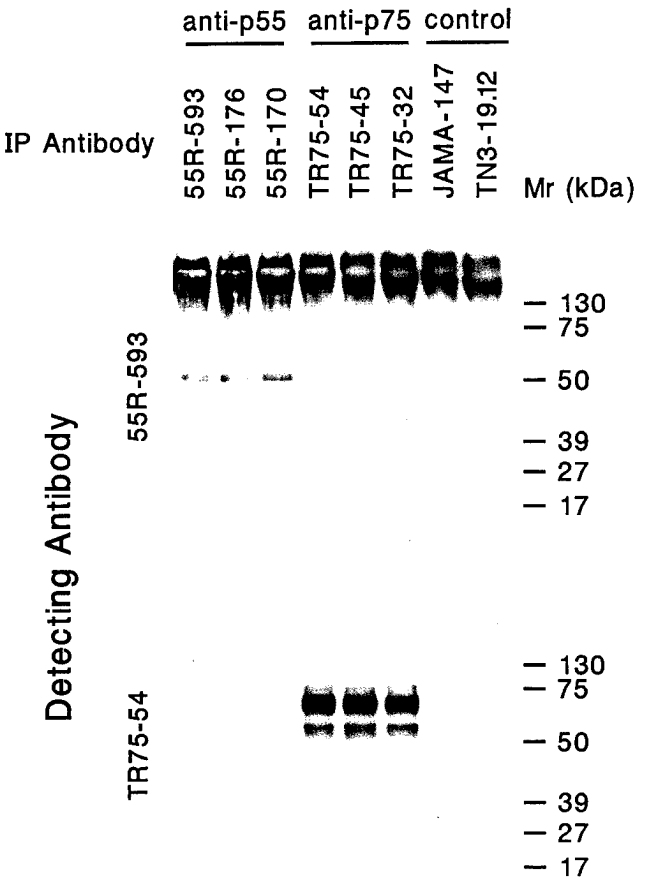

Figure 1. Immunoprecipitation of TNF receptors from L929 cells with anti-p55 and anti-p75 TNF receptor mAbs. Murine L929 lysates were incubated with 20 μg of p55- or p75-specific mAbs and protein A–Sepharose. Precipitates from 10^7 cells were separated by SDS-PAGE on 12% polyacrylamide gels under nonreducing conditions, transferred to nitrocellulose, and detected with 2 μg/ml either biotin-conjugated 55R-593 (*top*) or TR75-54 (*bottom*). Western blots were developed with streptavidin-HRP (1:4,000) and the ECL system.

results thus established that each family of mAb reacted only with the class of TNF receptor used for immunization and that the antibodies recognized the natural receptor molecules derived from whole cells.

Inhibition of Ligand Binding. Radioligand-binding studies were performed on murine L929 cells, which express both p55 and p75 TNF receptors to further characterize the specificity of each mAb. To monitor effects on the p55 TNF receptor, the antibodies were tested for their ability to inhibit binding of radiolabeled HuTNF to murine L929 cells since the human ligand binds exclusively to the murine p55 TNF receptor. Incubation of L929 with 55R-593, 55R-176, or 55R-170 produced a dose-dependent inhibition of binding of 4ng ^{125}I-HuTNF (Fig. 2 *A*). 50% inhibition of HuTNF binding was achieved at mAb doses of 4, 5, and 1,500 ng, respectively. No inhibition was detected with 55R-286, even at mAb doses >75 μg/well. Binding of HuTNF was not inhibited with the 6C8 control hamster IgG (Fig. 2 *A*) or p75-specific mAb (data not shown).

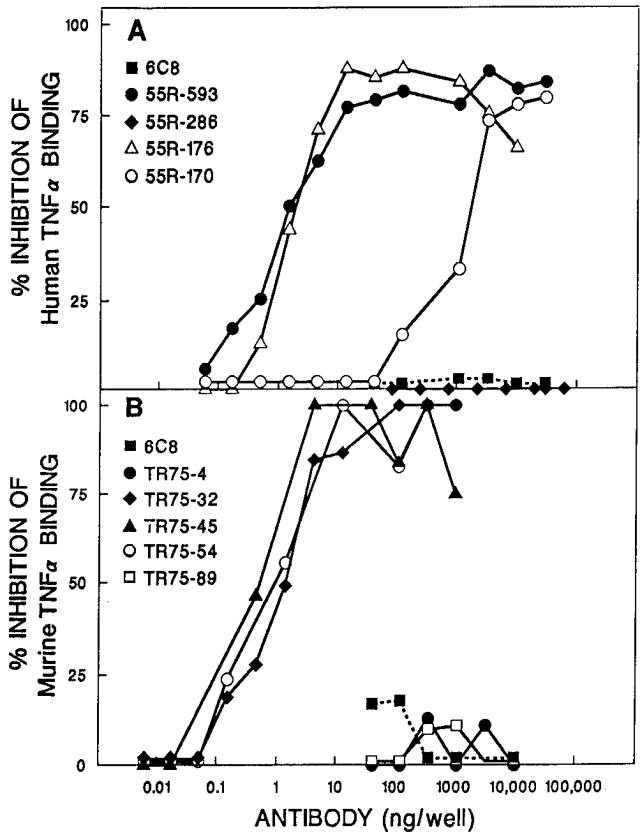

Figure 2. Inhibition of ligand binding by p55- or p75-specific TNF receptor mAbs. (*A*) L929 cells (4×10^6/well) were incubated with increasing concentrations of 55R-593, -286, -176, -170, or control hamster Ig 6C8 for 1 h at 4°C before addition of 4 ng radioiodinated HuTNF. Cell-associated and free radioactivity were separated by centrifugation through oil. Maximum binding of ^{125}I-HuTNF was 15,000 cpm, while nonspecific binding in the presence of 5 μg unlabeled HuTNF was <2%. (*B*) To assess p75-specific ligand binding, L929 cells were first incubated with saturating concentrations of HuTNF (5 μg) for 1 h at 4°C before addition of various doses of TR75-4, -32, -45, -54, and -89 or control hamster Ig 6C8. After 1 h at 4°C, 20 ng radioiodinated MuTNF was added for an additional hour. Samples were harvested as above and the percent binding inhibition determined. Binding of 20 ng labeled MuTNF yielded 5,000 cpm, and nonspecific binding in the presence of an additional 5 μg unlabeled MuTNF was 1.89%.

To assess the ability of the anti-p75 receptor mAb to inhibit ligand binding, L929 cells were pretreated with saturating concentrations (5 μg) of unlabeled HuTNF (to block all of the p55 receptors), followed by addition of ^{125}I-murine TNF, which thus bound exclusively to the p75 TNF receptor. Three p75-specific mAbs inhibited binding of ^{125}I-MuTNF in a dose-dependent manner (Fig. 2 *B*). 50% inhibition of binding of 20 ng radiolabeled MuTNF was achieved with 0.5, 0.9, and 1.5 ng of TR75-45, -54, and -32, respectively. No inhibition was observed with the TR75-4 and TR75-89 mAbs or control hamster IgG (6C8). Thus, each family of murine TNF receptor–specific reagents contained both receptor blocking and nonblocking mAbs.

Effect of p55 and p75 mAbs on In Vitro Cellular Responses to TNF. Fig. 3, *A* and *B*, depict the results of experiments to determine the agonist activity of the TNF receptor–specific mAb in the in vitro L929 killing assay in the absence of exogenous TNF. Only 55R-593 induced L929 cell lysis in a dose-dependent fashion (Fig. 3 *A*). However, addition of goat anti–hamster Ig to cultures pretreated with 55R-170 and -176 induced L929 lysis, while cross-linking of 55R-286–treated cultures or cultures treated with the 6C8 control mAb failed to induce cell killing (data not shown). The efficacy of 55R-593–induced L929 killing was not affected by antibody cross-linking. None of the anti-p75 mAbs induced cytolytic activity when used alone (Fi. 3 *B*), in combination, or when cross-linked with anti–hamster Ig (data not shown). These results thus demonstrate that 55R-593 displays TNF agonist activity and confirms that L929 cell-killing activity can be effected solely through p55 receptor engagement, but not p75 engagement.

The antibodies were also tested for their capacity to inhibit TNF-dependent L929 killing. The p55-specific mAbs 55R-170 and -176 blocked the lytic activity of 30 pg MuTNF in a dose-dependent fashion. 50% inhibition of TNF activity was obtained with 1,000 and 20 ng of the mAbs, respectively (Fig. 3 *C*), and corresponds to their relative abilities to inhibit ligand–receptor interaction. The p55 agonist mAb 55R-593, the nonblocking p55 mAb 55R-286, and control mAb failed to inhibit MuTNF-dependent cytolytic activity. When the experiments were conducted with HuTNF (which binds only to the murine p55 TNF receptor), similar results were obtained, except that 55R-170 and -176 were more efficient inhibitors (ID_{50} = 35 and 2 ng, respectively) (Fig. 3 *E*). Thus, mAbs 55R-170 and -176 act as TNF antagonists in the L929 killing assay. When similar experiments were performed with anti-p75 mAbs, partial inhibition of MuTNF lytic activity (up to ~60%) was noted (Fig. 3 *D*). However, the anti-p75 mAbs, even at doses of 30 μg/well, failed to inhibit L cell killing mediated by HuTNF (Fig. 3 *F*). An identical pattern of results was obtained when these two mAb families were tested for their ability to induce or inhibit TNF-mediated nitric oxide production (data not shown).

To examine whether the anti-p75 mAbs affected biologic responses mediated solely by the p75 TNF receptor, we used an assay that monitors murine TNF–induced proliferation of the murine CT6 cytotoxic T cell line. This cell line proliferates in response to polyvalent p75-specific antisera, but not p55-specific antisera (11). While none of the p75-specific mAbs elicited a proliferative response alone, all of the p75-specific mAbs induced significant CT6 expansion when cross-linked with goat anti–hamster Ig (data not shown). None of the p55-specific mAbs, either alone or after cross-linking, induced proliferation of CT6. These data thus confirm that clustering of the p75 TNF receptor is sufficient to induce a proliferative response in these cells. In contrast, TR75-45, -54, and -32 inhibited CT6 proliferative responses induced by 2 ng MuTNF in a dose-dependent manner (Fig. 4 *B*). The ID_{50} values for each mAb (2, 5, and 22 ng, respectively) correlated with the ability of each mAb to block ligand–receptor interaction. TR75-4 and -89 did not display functional inhibitory activity. Antibodies reactive with p55 TNF receptor had no significant inhibitory effect on TNF-dependent CT6 proliferation (Fig. 4 *A*).

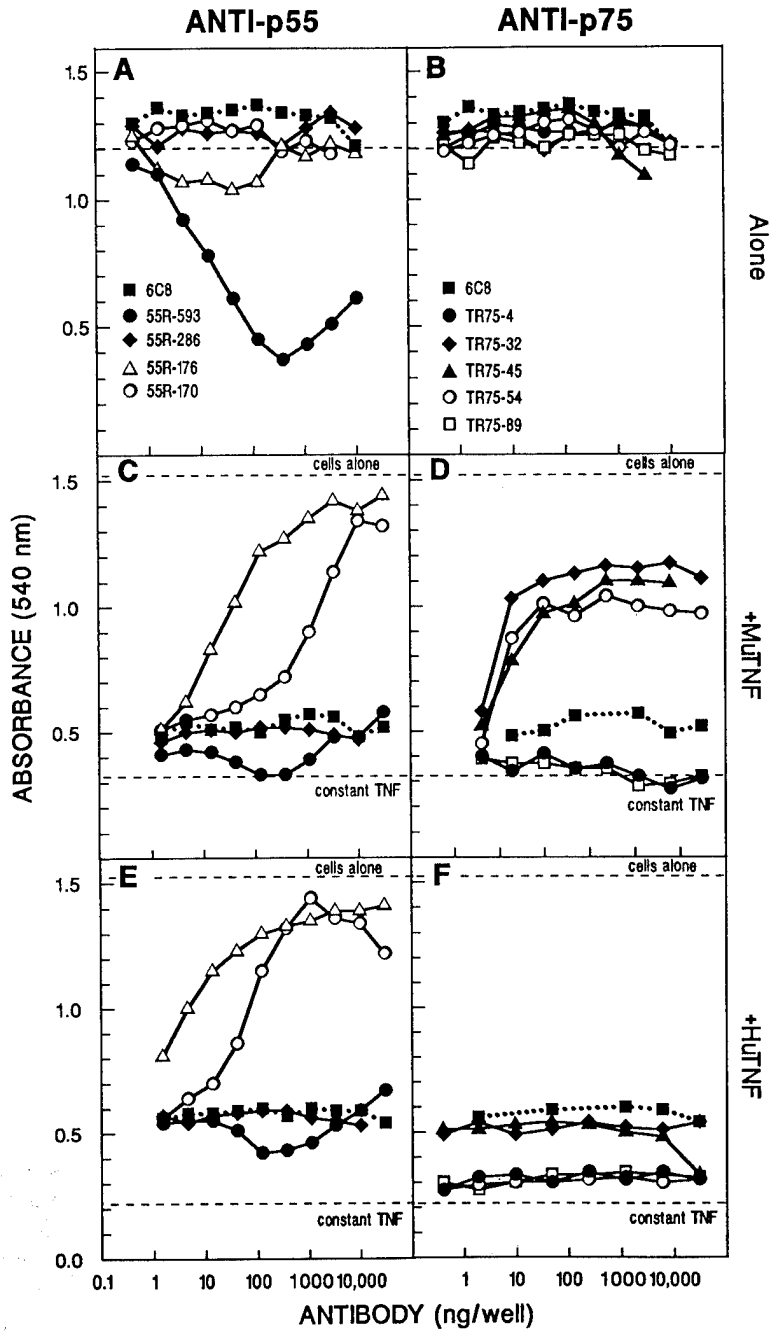

Figure 3. Agonist/antagonist activity of TNF receptor mAbs for L929 killing. Various dilutions of anti-p55 (*A*, *C*, and *E*), anti-p75 (*B*, *D*, and *F*) or control Ig 6C8 were preincubated with actinomycin D–treated L929 cells for 2 h at 37°C before addition of either medium (*A* and *B*) or 30 pg/well MuTNF (*C* and *E*) or 700 pg/well HuTNF (*D* and *F*). Monolayer viability was assessed after 18 h by vital dye uptake. Untreated control monolayers exhibited an absorbance at 540 nm of 1.2 (*A* and *B*) or 1.5 (*C–F*). Incubation with murine or human TNF resulted in readings of 0.32 and 0.22, respectively.

Effects of p55 and p75 mAb In Vivo. Pharmacokinetic studies performed on each antibody in naive BALB/c mice established that all mAbs displayed serum half-lives of ~2 d (data not shown). The half-life of each antibody remained unchanged in mice that received four mAb injections over a 16-wk period. Thus the hamster anti-TNF receptor mAbs were not immunogenic in mice.

To examine the efficacy of the receptor-specific mAbs in vivo, we initially used two experimental systems (LPS/galactosamine–mediated lethal shock and resistance to infection by *L. monocytogenes*), which, based on experiments with p55-deficient mice, are known to be obligatorily dependent on p55 TNF receptor engagement (21, 22). Animals pretreated with either saline or control hamster IgG succumbed to the lethal combination of LPS and galactosamine within 24 h (Fig. 5 *A*). Pretreatment of mice with 250 μg TN3-19.12 (a neutralizing hamster mAb specific for murine TNF-α) completely protected mice from lethal shock. Mice pretreated with the antagonistic 55R-170 mAb were also protected from lethal shock. Similar results were obtained by use of the other p55 antagonist mAb 55R-176 (data not shown). In separate dose–response experiments, 40 μg of 55R-170 was sufficient to provide complete protection (data not shown). In contrast, the antagonistic anti-p75 TNF receptor mAb TR75-54 was not protective at any dose.

In the *Listeria* infection model, mice treated either with

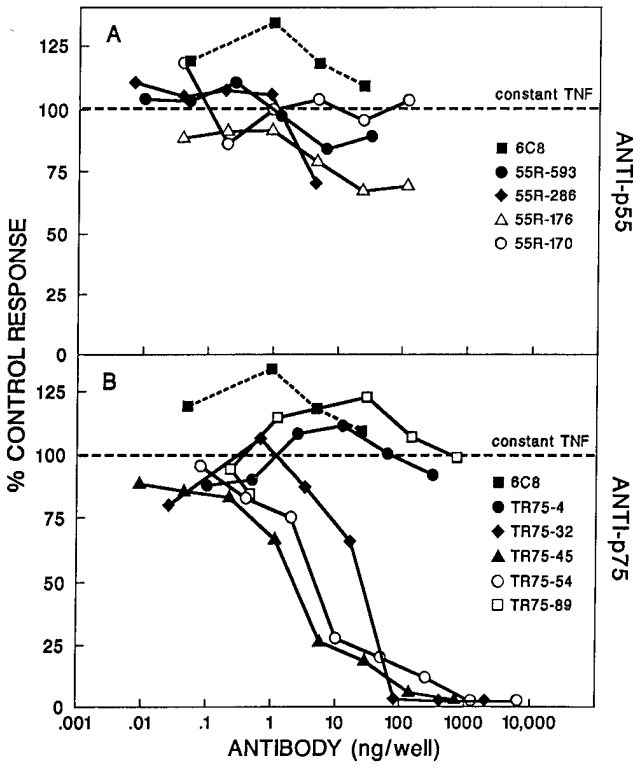

Figure 4. Anti-p75 inhibition of TNF-mediated CT6 proliferation. CT6 cells (5×10^4) were incubated with anti-p55 (*A*), anti-p75 (*B*), or control hamster mAb 6C8 for 2 h at 37°C before addition of 2 ng MuTNF. Cultures were pulsed after 20 h with [^{3}H]thymidine for an additional 4–6 h and the percent of control response determined. Cultures stimulated with 2 ng MuTNF alone yielded 12,300 cpm with background counts <3,500.

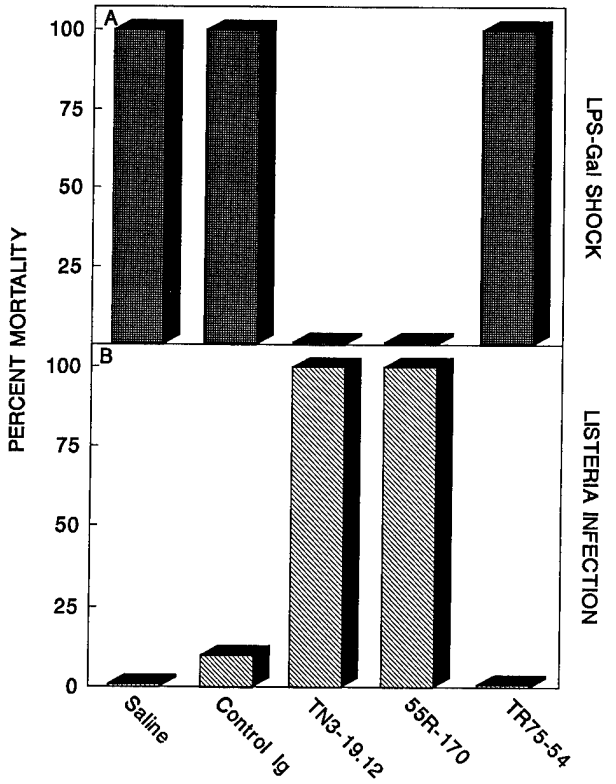

Figure 5. In vivo activity of TNF receptor–specific mAbs. (*A*) LPS/galactosamine–mediated lethal shock. Groups of 10 C57Bl/6 mice were treated with saline or 250 μg mAb 24 h before challenge with a combination of LPS (600 ng) and D-galactosamine (10 mg). Survival was monitored over a 48-h period. (*B*) *Listeria* infection. Groups of 5 C3H/HeJ mice were injected intraperitoneally with 250 μg antibody 6 h before infection with 3,000 live *Listeria* organisms. Survival was monitored twice daily over a 10-d period. Depicted is one of six representative experiments.

saline or irrelevant hamster mAb mounted a protective anti-*Listeria* response and survived infection with 3,000 live *Listeria* organisms (Fig. 5 *B*). Mice treated with anti-TNF became susceptible to lethal infection and died. Animals treated with the antagonist p55-specific mAb (55R-170) failed to generate an anti-*Listeria* response and succumbed to the infection. As little as 25 μg of 55R-170 was sufficient to render all the animals susceptible to infection (data not shown). In contrast, TR75-54 did not inhibit the development of an anti-*Listeria* response in mice. Subsequent testing of TR75-32, -45, and -54 at doses of 250 and 500 μg per mouse showed that none of the p75-specific mAbs affected development of anti-*Listeria* responses (not shown). Thus, in both in vivo models, anti-p55–treated mice responded similarly to mice lacking the p55 TNF receptor. Moreover, antibody blockade of p75 was without functional consequences.

p75 TNF Receptor Engagement Is Required for MuTNF-mediated Skin Necrosis. Whereas the aforementioned studies confirmed the physiologic requirement of p55 TNF receptors in promoting protective and immunopathologic responses to bacteria and their products in vivo, no information was obtained that helped define the physiologic role of p75 TNF receptors. Insights into p75's biologic role in vivo were gained from experiments being conducted to explore TNF's immunosuppressive activities. In these studies, daily subcutaneous injections of either murine or human TNF into mice led to a state of immunosuppression that blocked xenograft rejection (41) and inhibited T cell priming to the soluble protein antigen, hen egg lysozyme (Sheehan, K., and R. Schreiber, unpublished observations). During the course of these experiments, we observed that subcutaneous injection of 3 μg MuTNF produced severe hemorrhage in the skin and subsequent necrosis at the injection site (Fig. 6 *B*), while s.c. injection of 3 or 30 μg HuTNF did not (Fig. 6 *C*). In CBA/J mice, the lesion induced with MuTNF was observed after three to five daily injections. The lesion initially presented as a reddening of the skin, followed by intradermal hemorrhage and skin necrosis. Histologically, the full-thickness skin and subcutaneous sections of the saline- and HuTNF-treated mice disclosed an absence of microscopic abnormalities (Fig. 7, *A* and *C*) to correspond with the unremarkable gross features depicted in Fig. 6, *A* and *C*. In contrast, early epidermal necrosis, transdermal neutrophilia and hemorrhage, subcutaneous hemorrhage, and recent thrombi were present in the MuTNF-treated mice (Fig. 7 *B*, *inset*). This result thus suggested that the p75 TNF receptor was involved in this process.

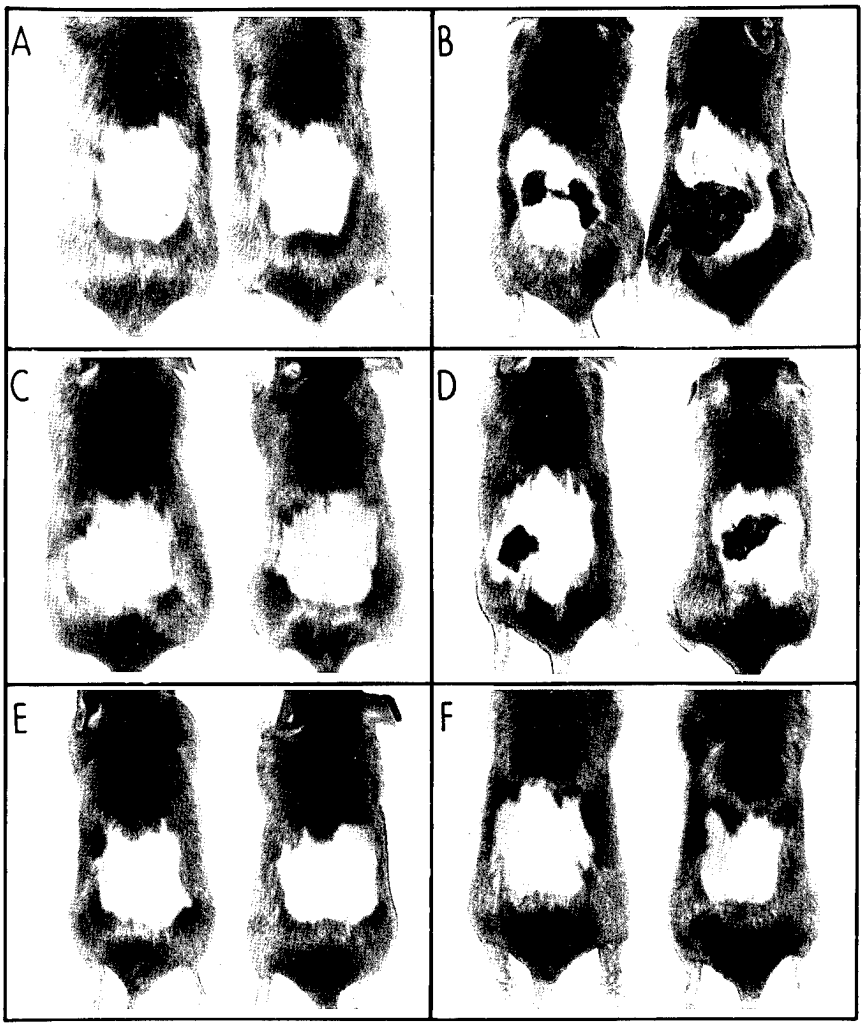

Figure 6. Prevention of MuTNF-induced skin necrosis by anti-p55 and anti-p75 TNF receptor–specific mAbs. Mice were pretreated on day −1 with either saline or 100 μg of antibody intraperitoneally and injected daily with 3 μg TNF alone or combined with 50 μg mAb subcutaneously over a period of 7 d. (*A*) Saline alone; (*B*) MuTNF only; (*C*) HuTNF only; (*D*) MuTNF + 6C8 control IgG; (*E*) MuTNF + 55R-170; and (*F*) MuTNF + TR75-54.

To further confirm the roles of the p55 and p75 TNF receptors in this process, mice were treated with MuTNF either alone or in combination with control or antagonistic p55- or p75-specific mAbs. None of the mAbs individually or collectively induced the skin necrosis reaction (data not shown). Pretreatment of mice with antagonistic p75 mAbs (TR75-32 or -54) completely blocked the development of the skin reaction (Fig. 6 *F*) as well as any histologic changes (Fig. 7 *F*). Similar results were obtained by use of the p55-specific antagonistic mAb 55R-170 (Figs. 6 *E* and 7 *E*). Control hamster Ig was without effect, resulting in a composite of microscopic changes virtually identical to those in mice treated with MuTNF alone (Fig. 7 *B*) with epidermal necrosis, hemorrhage, neutrophilia, and thrombosis (Figs. 6 *D* and 7 *D*). These results thus demonstrate, for the first time, an in vivo role for the p75 TNF receptor and suggest that p75 engagement may be directly involved in certain types of acute inflammatory and hemorrhagic reactions.

Discussion

The studies presented here document the production of hamster mAbs specific for the extracellular domains of the p55 and p75 murine TNF receptors. These reagents represent the first murine TNF receptor–specific mAbs to be generated. Each member of the anti-p55 or anti-p75 mAb family displayed strict specificity for the receptor form used for immunization. Within the anti-p55 family, 55R-286 did not interfere with p55-TNF interaction, while 55R-170, -176, and -593 blocked the ability of p55 to bind ligand in a dose-dependent manner. Three p75-specific mAbs (TR75-32, -45, and -54) were identified that blocked ligand–receptor interaction, while two others (TR75-4 and -89) did not.

Functionally, the antibodies could be distinguished by their effects in well-defined in vitro TNF bioassays. By use of the L cell–killing assay, the anti-p55 mAb family was found to contain one mAb (55R-593) that acted as a TNF agonist, two (55R-170 and -176) that acted as antagonists, and one (55R-286) that did not affect TNF's actions on p55. The production of an agonistic anti-murine p55 TNF receptor mAb is notable because its functional activity makes it unique among anti-p55 mAbs. Although it has been shown that signaling through the p55 TNF receptor is initiated by oligomerization of surface receptors using either polyvalent anti-p55 sera (11, 42) or homotrimeric ligand (43), the activity of the 55R-593 mAb presented in this report suggests that dimerization of the p55 TNF receptor may be sufficient to

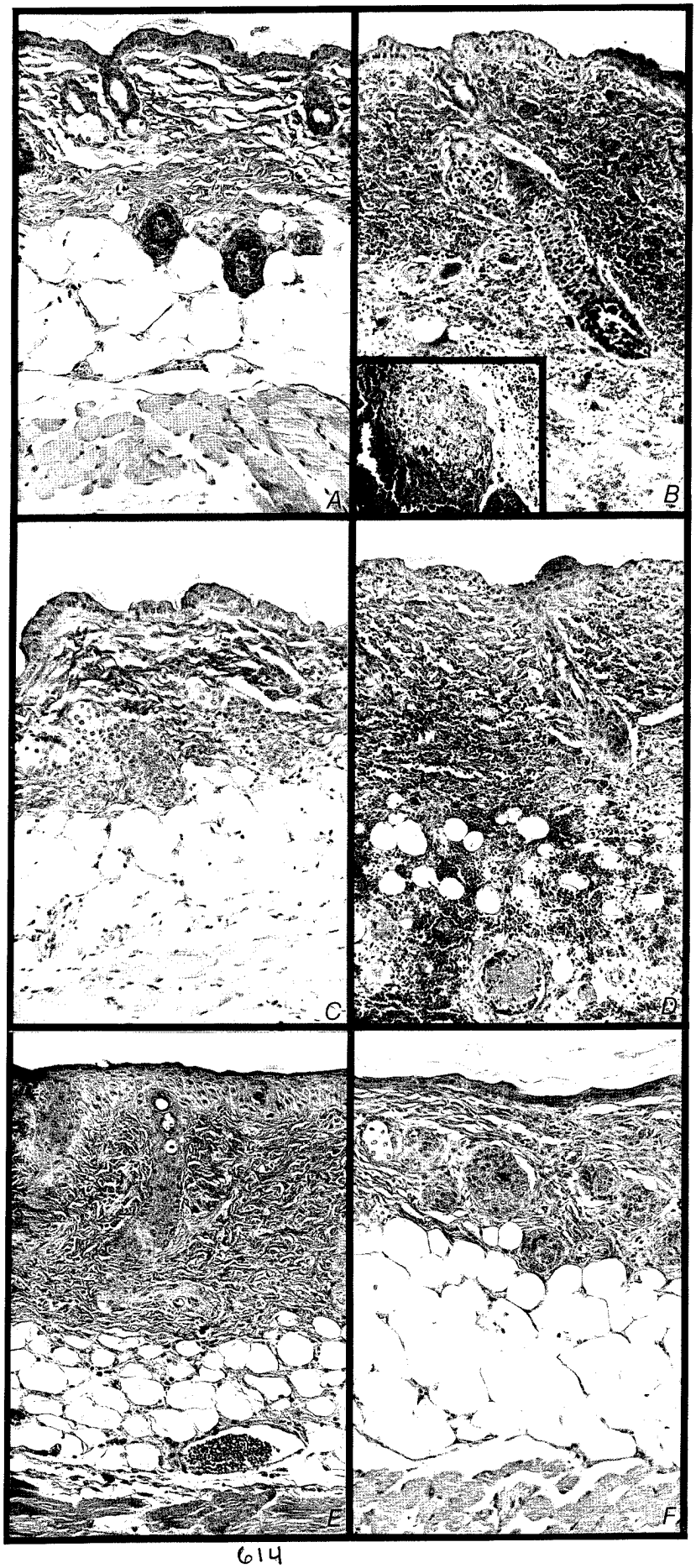

Figure 7. Histologic examination of TNF-mediated skin necrosis. Mice were treated as above with 100 μg antibody intraperitoneally on day −1 and 3 μg TNF alone or in combination with 50 μg antibody subcutaneously daily. After 4 d, mice were killed and tissue processed and stained with hematoxalin and eosin. In vivo treatments are as follows: (*A*) saline only; (*B*) MuTNF alone; (*C*) HuTNF alone; (*D*) MuTNF + 6C8 control IgG; (*E*) MuTNF + 55R-170; and (*F*) MuTNF + TR75-32. ×200.

induce a biologic response in these cells. Importantly, the antagonistic p55-specific mAb could be converted into agonists after cross-linking. In fact, in early experiments, small amounts of high molecular weight mAb aggregates present in the purified antibody preparations led to an agonist action of 55R-170 and -176. Addition of a gel filtration step to the mAb purification procedure separated the high molecular mass aggregates that displayed potent agonist activity from the bulk of the monomeric mAbs that displayed antagonistic activity. When 55R-593 was subjected to the same gel filtration procedure, the agonist activity coeluted with monomeric antibody, con- firming that 55R-593 acted as an agonist in its native form. Thus, these results not only document the functional activity of the p55-specific mAbs, but also confirm the concept that engagement of p55 is both necessary and sufficient to induce killing of L929 cells.

The same assays were used to show that the anti-p75 mAb family contained three members that acted as p75 antagonists (TR75-32, -45, and -54) and two that were unable by themselves to affect TNF-dependent biologic responses in cells (TR75-4 and -89). By use of the CT6 proliferation assay, TR75-32, -45, and -54 were found to inhibit MuTNF-driven proliferative responses in a dose-dependent manner, while TR75-4 and -89 did not. These mAbs represent the only reagents currently available that display antagonist activity for the murine p75 TNF receptor and thus document that engagement of p75 is both necessary and sufficient to induce proliferative responses in CT6 cells.

The p75 antagonist mAbs also partially inhibited MuTNF-dependent L cell killing. Inhibition was never complete (40–60%) and could be overcome if higher concentrations of murine TNF were used. Moreover, none of the anti-p75 reagents effected L cell killing when cross-linked in the absence of TNF. These results thus best support the model where p75 plays an accessory role in the killing process. The precise role of p75 in mediating L cell killing remains controversial. Heller et al. (23, 24) proposed that p75 can directly signal for cytotoxicity, while Tartaglia et al. (12, 30) proposed that p75 facilitates ligand uptake onto p55 and that p55 is the sole TNF receptor responsible for initiating the L cell–killing response. Recently, the latter "ligand-passing model" has been supported by the results of structural analyses of the two TNF receptors' intracellular domains. The p55 intracellular domain contains a "death domain" similar to that found in the intracellular domain of the Fas antigen that is important in effecting cell killing (44), while the p75 TNF receptor does not. Moreover, additional support for a ligand-passing function has been derived from the characterization of mice with selective genetic deficiencies of either the p55 (21, 22) or p75 (44a) TNF receptors.

To initially examine the potential usefulness of the anti-p55 and -p75 mAbs in vivo, we used two well-characterized murine models (39, 45) that are known to be unequivocally dependent on endogenous TNF production. As little as 25 or 40 μg of antagonistic anti-p55 inhibited development of a protective immune response to *L. monocytogenes* in naive mice and protected normal mice from the lethal effects of LPS/galactosamine, respectively. In contrast, up to 500 μg quantities of antagonistic p75 TNF receptor–specific mAbs were without biologic effects. Importantly, these results recapitulate the observations made by others using mice lacking p55 (21, 22) or p75 (44a) and thereby validate the in vivo effects of our anti-p55 and anti-p75 mAbs.

The characterization of the specificities and functional activities of the murine TNF receptor–specific mAbs was based on the known structure and function of the two receptors. Whereas definitive experiments have now been performed that define at least some of the physiologic roles of the p55 TNF receptor as they occur in vivo, little information is available concerning the physiologic function of the p75 TNF receptor. The availability of antagonistic mAbs specific for the murine p75 TNF receptor has permitted us to identify a heretofore unrecognized role of this protein in promoting inflammatory responses. Previous studies by others (46) have indicated that TNF plays an important role in the development of naturally occurring cutaneous diseases. Moreover, subcutaneous injection of highly purified recombinant TNF into the skin of normal mice and rabbits has been shown by some groups to induce intradermal hemorrhage and skin necrosis (47, 48). In studies in which human TNF was administered to experimental animals, the development of the cutaneous inflammatory response was found to require the additional presence of bacterial products (49), whereas repetitive subcutaneous administration of LPS-free murine TNF to mice led to generation of clear necrotic skin lesions (46, 48).

Based on the recognized differential species specificity displayed by the p55 and p75 murine TNF receptors for human versus murine TNF, the previous discrepant results suggested that both p55 and p75 TNF receptors might be involved in the process. In this report, we show that whereas repeated injections of MuTNF induced frank intradermal hemorrhage and severe skin necrosis, repeated injections of up to 10 times larger quantities of human TNF were without effect. This result suggested that p75 was involved in the inflammatory skin reaction. This hypothesis was supported by our observation that TNF-induced skin necrosis could be inhibited by administration of antagonistic p75-specific mAbs. Moreover, since skin necrosis was also inhibited by blocking p55-specific mAbs, the ultimate development of in vivo inflammatory responses in the skin must be due to engagement of both TNF receptor forms.

At this time, the identity of the cells whose function is regulated by the p75 and p55 TNF receptors remains unknown. It is also unclear whether the dual requirement for p55 and p75 in this process involves a single cell population that is stimulated by engagement of both types of TNF receptors or multiple cell populations that are specifically induced to perform their function through engagement of one or the other TNF receptors. The gross and histologic findings in this study, in particular the presence of neutrophilia, intense hemorrhage, and thrombosis, would suggest that endothelial cells and neutrophils may be important cellular participants in this model. It is noteworthy that, based on in vitro experiments, others (50) have suggested that p75 may be important in effecting biologic response induction in endothelial cells. Studies are currently underway in the labora-

tory to define the precise physiologic roles of p55 and p75 in promoting inflammatory reactions.

Thus, we have documented the production and antigen-binding characteristics of two novel families of hamster mAbs specific for the murine p55 and p75 TNF receptors. The ability of these mAbs to function in vitro and in vivo coupled with their lack of immunogenicity in vivo provides the opportunity to modulate TNF-mediated responses in a receptor-specific fashion. The availability of these monoclonal murine TNF receptor–specific reagents will thus facilitate investigations aimed at defining the molecular basis for TNF's pleiotropic actions and the physiologic roles of the two TNF receptors in vivo.

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Note Added in Proof: While this paper was in press, the paper describing the generation and characterization of p75 TNF receptor deficient mice, referred to in the Discussion, by Erickson et al. (44a), was accepted and published.

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